NF-κB Links CO2 Sensing to Innate Immunity and Inflammation in Mammalian Cells

Eoin P. Cummins, Kathryn M. Oliver, Colin R. Lenihan, Susan F. Fitzpatrick, Ulrike Bruning, Carsten C. Scholz, Craig Slattery, Martin O. Leonard, Paul McLoughlin and Cormac T. Taylor

J Immunol 2010; 185:4439-4445; Prepublished online 3 September 2010;
doi: 10.4049/jimmunol.1000701
http://www.jimmunol.org/content/185/7/4439

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/09/03/jimmunol.100070.1.DC1

References
This article cites 26 articles, 11 of which you can access for free at:
http://www.jimmunol.org/content/185/7/4439.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
NF-κB Links CO2 Sensing to Innate Immunity and Inflammation in Mammalian Cells

Eoin P. Cummins, Kathryn M. Oliver, Colin R. Lenihan, Susan F. Fitzpatrick, Ulrike Bruning, Carsten C. Scholz, Craig Slaterry, Martin O. Leonard, Paul McLoughlin, and Cormac T. Taylor

Molecular O2 and CO2 are the primary substrate and product of aerobic metabolism, respectively. Levels of these physiologic gases in the cell microenvironment vary dramatically both in health and in diseases, such as chronic inflammation, ischemia, and cancer, in which metabolism is significantly altered. The identification of the hypoxia-inducible factor led to the discovery of an ancient and direct link between tissue O2 and gene transcription. In this study, we demonstrate that mammalian cells (mouse embryonic fibroblasts and others) also sense changes in local CO2 levels, leading to altered gene expression via the NF-κB pathway. IKKα, a central regulatory component of NF-κB, rapidly and reversibly translocates to the nucleus in response to elevated CO2. This response is independent of hypoxia-inducible factor hydroxylases, extracellular and intracellular pH, and pathways that mediate acute CO2-sensing in nematodes and flies and leads to attenuation of bacterial LPS-induced gene expression. These results suggest the existence of a molecular CO2 sensor in mammalian cells that is linked to the regulation of genes involved in innate immunity and inflammation. The Journal of Immunology, 2010, 185: 4439–4445.

Changes in intracellular O2 are sensed by a family of proline and asparagine hydroxylases leading to altered gene transcription via the hypoxia-inducible factor (HIF), which governs the adaptive response to hypoxia (1, 2). In earlier work, we demonstrated that NF-κB, a master regulator of innate immune and inflammatory gene expression, is induced by hypoxia through a similar hydroxylase-dependent mechanism (3).

CO2 production is coupled to oxygen consumption. As a result, physiologic CO2 levels are higher in tissues than in the atmosphere and can change dramatically under conditions in which cellular metabolism is altered. Acute CO2 sensing has been reported in specialized cells in several lower animal species, including flies, nematodes, and rodents, leading to rapid neuronal signaling that directs responses as diverse as survival, avoidance, and olfactory sensation (4–6). However, little is known regarding the effect of altered CO2 on gene expression.

Permissive hypercapnia, which occurs when blood pCO2 is elevated during hypoventilation of intubated patients, attenuates mortality during acute respiratory distress syndrome (7). Similarly, hypercapnic acidosis attenuates endotoxin-induced acute lung injury, supporting a generally anti-inflammatory effect of CO2 (8).

Furthermore, elevated CO2 increases mortality in Drosophila exposed to pathogens and augments infection-induced injury in rats, indicating a role for CO2 sensing in suppression of innate immunity (9, 10). NF-κB is a key transcriptional regulator of inflammation and innate immunity (11). Recent studies have implicated altered NF-κB signaling in the attenuation of inflammatory gene expression by hypercapnic acidosis and in the regulation of the Drosophila NF-κB homolog Relish by CO2 (9, 12). In this study, we describe a CO2-dependent nuclear localization of the NF-κB signaling protein IKKα. The sensitivity of IKKα to CO2 is rapid, reversible, and dose dependent. It is independent of the HIF signaling pathway, pathways involved in CO2 sensing in lower species, extracellular pH, and intracellular pH. The CO2-dependent nuclear localization of IKKα is associated with a marked attenuation of NF-κB signaling, with effects evident at the level of cytoplasmic IkBα, nuclear p65, and NF-κB target gene expression.

Materials and Methods

Cell culture

Mouse embryonic fibroblasts (MEFs), HK-2, Hela, CCD-19Lu, A549, and primary human PBMCs were cultured at 21% O2 and 5% CO2 and maintained in a humidified tissue culture incubator prior to exposure to the conditions indicated in the individual experiments. Media used in each case were specific to each cell type. All reagents were produced by Life Technologies (Rockville, MD) unless indicated otherwise. MEF media consisted of DMEM high glucose with L-glutamine supplemented with 10% FCS, and penicillin/streptomycin (P/S). Hela and CCD-19Lu media consisted of minimal essential medium, 10% FCS, P/S, L-glutamine, and nonessential amino acids (Sigma-Aldrich, St. Louis, MO). HK-2 media consisted of DMEM-Hams F12 (1:1; Sigma-Aldrich) P/S, L-glutamine, and 10 mg/ml epidermal growth factor + supplements. PBMC media consisted of medium 199/Earle’s balanced salt solution + Earle’s balanced salts + L-glutamine (Thermo Environmental Instruments, Franklin, MA), P/S, 10% human serum, and polymixin B sulfate (10 μg/ml).

PBMC isolation and culture

Whole blood was drawn from human volunteers and immediately added to a BD Vacutainer CPT (Becton Dickenson, Franklin Lakes, NJ) cell preparation tube with sodium citrate. Blood was centrifuged within 30 min of drawing and was centrifuged at 1500 relative centrifugal force for 20 min.
Mononuclear fraction of cells was isolated and washed two times with 10–15 ml PBS with centrifugation at 300 relative centrifugal force for 15 min in between washes. Cells were then resuspended in appropriate media in flasks. Media were changed on days 4 and 8. The experiment was performed on day 9.

**Media types used in the experiments**

Hypoxia experiments. Cell-specific media, as indicated above, were used for each cell type for the hypoxia-graded oxygen experiments (Fig. 1A, 1B, Supplemental Fig. 1A, 1B).

Hypercapnia experiments. MEF media diluted (9:1) with 100 mM HEPES (pH 6.8; Sigma-Aldrich) was used for the initial hypercapnia experiments (Figs. 1C, 2–4, Supplemental Fig. 1C).

Buffered hypercapnia experiments. MEF media diluted (9:1) with 250 mM HEPES (pH 7.5) and supplemented with concentrated HCl or NaCl (to correct for osmolarity change) and equilibrated overnight at 0 or 10% CO₂ prior to experimentation (Figs. 1D–E, 5, 7, Supplemental Figs. 3, 5–7).

Graded pH experiments. DMEM high-glucose powder supplemented with HEPES [Sigma-Aldrich no. D2903 (Fig. 6) and Sigma-Aldrich no. D1152 (Supplemental Fig. 4)] was reconstituted, filter-sterilized, and supplemented with FCS (10%) and P/S. A range of NaHCO₃ (Sigma-Aldrich) concentrations was prepared in the media for the distinct media compositions (a range of NaCl [Sigma-Aldrich] concentrations was supplemented to correct for osmolarity differences between the media types). Media were equilibrated for 4 h at 0.03 or 10% CO₂ prior to experimentation (Fig. 6, Supplemental Fig. 4).

**Preparation of cytosolic and nuclear fractions**

Cells were grown for the indicated time period in normoxia, hypoxia, or hypercapnia in either an open environment or a closed chamber. In the case of cells within closed chambers, extracts were prepared within the chamber. The media were aspirated and cells placed on ice. Cells were then washed briefly in ice cold PBS. The PBS was then aspirated, and 200 μl cytosolic extract lysis buffer. Buffer A (10 mM HEPES [pH 8], 1.5 mM MgCl₂, 10 mM KCl, 200 mM sucrose, 0.5 mM DTT, 0.25% NP-40 (JPEGA) + protease inhibitor mixture) was added to the Petri dish (100 mm). Cells were left to swell for 5–10 min on ice before being scraped with a rubber policeman. Lysate was then centrifuged at 12,000 rpm for 1 min at 4°C. The supernatant is the cytosolic fraction. Cytosolic extract was stored at −20°C. The pellet was then washed with 500 μl buffer A and centrifuged again at 12,000 rpm for 1 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 100 μl nuclear extract lysis buffer and buffer C (20 mM Hepes [pH 8], 420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 0.5 mM DTT, 25% glycerol + protease inhibitor mixture) and was rocked on ice for 30 min. The 1.5 ml Eppendorf tube was briefly vortexed before centrifuging at 14,000 rpm for 10 min at 4°C. The supernatant contains the nuclear fraction. Nuclear fraction was stored at −80°C. Fractionation was validated by enrichment of a predominantly cytosolic protein InxBo in the cytosolic fraction and enrichment of the nuclear protein lamin A/C in the nuclear fraction, as well as enrichment of p65 in the nuclear fraction following ligand activation.

**Immunoblotting**

Western blotting of cytosolic and nuclear fractions was performed according to standard protocols using the following primary Abs IKKalpha (no. 2682), IKKβ (no. 2370) lamin A/C (no. 2032), InxBo (no. 4814; Cell Signaling Technology, Beverly, MA), p65 (no. sc-372; Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (Sigma-Aldrich), HIF-1α (HA67) (Calbiochem, San Diego, CA) Tαa box binding protein (Abcam, Cambridge, MA).

**Transient transfection**

Transient transfection of plasmid DNA was performed in subconfluent cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) transfection reagent according to the manufacturer’s instructions. After transfection, cells were centrifuged on an NF-κB–luciferase reporter construct (Promega no. E849A). Cell lysates were prepared by using a 1× luciferase lysis buffer (Promega, Madison, WI). Substrate was added to the cell lysate and luciferase activity was measured using a desk-top luminometer (Berthold Technologies, Bad Wildbad, Germany). Experiments were performed in duplicate, and luciferase values were normalized to cotransfected β-Gal control vector activity.

**Fluorescent microscopy**

Cell fixation and immunostaining. MEF cells were grown on glass coverslips and exposed to 0.03 or 10% CO₂ for the indicated times. The cells were then washed twice with PBS and fixed with 3% paraformaldehyde (solutions were preequilibrated to the respective CO₂ environment); this was followed by additional washes with PBS and the quenching of free aldehyde groups with 50 mM ammonium chloride-PBS. The cells were washed with PBS as before and permeabilized with 0.1% Triton 100. After two more PBS washes, the cells were incubated in primary Ab diluted in 5% FCS/PBS (IKKα/1:50, Santa Cruz Biotechnology) in a closed humid chamber for 1 h. Afterward, cells were washed with PBS as before and incubated with a secondary Ab (Alexa Fluor 568 goat anti-rabbit IgG 1:100, Invitrogen) diluted in 5% FCS/PBS in a closed humid chamber for 1 h. The fixed cells were then washed as before and rinsed in distilled water before inversion and mounting on a glass slide (DakoCytomation fluorescent mounting medium; DakoCytomation, Carpenteria, CA). The slides were then protected from light overnight before imaging.

**Confocal imaging**

Confocal imaging was performed using a Carl Zeiss (Göttingen, Germany) LSM510 UVMETA system mounted on an Axiovert 200M computer-controlled microscope. Alexa Fluor 568 was excited using a 543-nm laser line from a helium-neon laser, and the images were acquired with a 63× Plan-Apo oil-immersion objective.

**Real-time PCR**

**RNA extraction.** Total RNA was prepared from cell using Trizol (Qiagen, Valencia, CA) and isopropanol precipitation or RNaseasy spin columns (Qiagen) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed using random primers and Superscript II reverse transcriptase according to the manufacturer’s instructions (Invitrogen).

**Quantitative real-time PCR.** Real-time PCR was performed on an Applied Biosystems 7900HT (Foster City, CA) fast real-time PCR system using mouse primers (full-reversed followed by for human primers (SABiosciences, Frederick, MD). The values obtained were normalized to 18S or β-actin and calculated according to the ACT method.

**PCR array.** A549 cells were exposed to ambient or 10% CO₂ for 4 h ± lymphotixin α1/β2 (100 ng/ml). cDNA was generated from RNA and incubated on an NF-κB Signaling pathway RT² qPCR array (5Biosciences) according to the manufacturer’s instructions. This array contains 84 key genes involved in the NF-κB signal transduction pathway and several controls. Target genes were normalized to control β-actin expression and expressed as fold change relative to ambient CO₂ control.

**Intracellular pH BCECF assay**

MEF were washed in serum free media and loaded with 5 μM BCECF-AM (Molecular Probes, Eugene, OR) in OptiMEM (Life Technologies) for 30 min at 37°C, 21% O₂, 5% CO₂. Dye was removed and cells were incubated in full DMEM media for 30 min at 37°C, 21% O₂, 5% CO₂. Cells were then exposed to pre-equilibrated neutral or acidic media at 0.03% CO₂ or 10% CO₂ for 1 h. Following exposure, cells were immediately assayed in a fluorescent plate reader at room temperature at 21% O₂ with ambient CO₂. The fluorophore was excited at 485 nm (A1) and 444 nm (A2), and emission was recorded at 538 nm in each case. The ratio A1/A2 is directly proportional to intracellular pH.

**Tryptan blue cell viability assay**

A549 cells were exposed to ambient or 10% CO₂ for 4 h. Culture media was collected and cells were washed once with PBS. PBS was collected and cells were trypanized using 10× Trypsin. Trypsinization was blocked using PBS media, and cell pellets were collected with a pipette. Cells were centrifuged at 4°C for 5 min at 1000 rpm. Pelleted cells were resuspended in full medium, and 10 μl of the resuspended cells was combined with 10 μl Tryptan blue solution. Viable and nonviable cells were counted on a hemocytometer and scored in duplicate for n = 3 experiments.

**Results**

We investigated the effects of altered atmospheric oxygen (1–21%) and carbon dioxide (0.03–5%) on NF-κB signaling in mammalian cells (Fig. 1A). MEFs were treated with dimethylxalloglycine
(DMOG; a pan-hydroxylase inhibitor) or an atmosphere of 1% O2, 5% CO2, with or without re-equilibration to ambient conditions (21% O2, 0.03% CO2). While nuclear HIF accumulation was detected with DMOG treatment or exposure to 1% O2, 5% CO2, nuclear IKKα accumulation was detected only in cells exposed to 1% O2, 5% CO2 (Fig. 1A) indicating that nuclear-translocation of IKKα is independent of hydroxylase-dependent oxygen sensing. This response occurred in multiple cell lines and was rapid and reversible with re-equilibration of cells to ambient conditions (Fig. 1A, Supplemental Fig. 1A, 1B).

To determine the relative contribution of O2 and CO2 to this response, we examined IKKα nuclear translocation in MEFs exposed to increasing atmospheric oxygen concentrations against a background of 5% CO2, without (H) and with (R) re-equilibration to ambient conditions. Reversible nuclear IKKα accumulation occurred in cells exposed to atmospheric oxygen concentrations up to 21% (with 5% CO2), leading us to hypothesize that the nuclear localization of IKKα is independent of changing O2 levels, but dependent on exposure to elevated CO2 (Fig. 1B).

To test this hypothesis, MEFs were exposed to increasing CO2 levels (0.03–10% with a balance of room air), and cells were either allowed to re-equilibrate to ambient CO2 (R) or retained at the indicated CO2 concentration at which nuclear lysates were prepared (H). Cells re-equilibrated to ambient CO2 demonstrated a loss of nuclear IKKα accumulation, thus confirming the existence of a rapidly reversible, CO2-dependent nuclear localization of IKKα (Fig. 1C). This response is evident over a range of physiologic CO2 concentrations (Supplemental Fig. 1D). Confocal microscopic analysis of cells exposed to 10% CO2 with and without re-equilibration to ambient CO2 confirmed the finding of reversible, CO2-dependent nuclear localization of IKKα (Fig. 1D). Our observations were further supported by a robust CO2-dependent nuclear localization of IKKα in primary human PBMCs exposed to 10% CO2 (Fig. 1E).

A number of regulatory roles for nuclear IKKα have been described (13–16). We investigated the effects of elevated CO2 on nuclear localization of IKKα in primary human PBMCs exposed to 10% CO2 (Fig. 1E).

A number of regulatory roles for nuclear IKKα have been described (13–16). We investigated the effects of elevated CO2 on nuclear localization of IKKα in primary human PBMCs exposed to 10% CO2 (Fig. 1E).

A number of regulatory roles for nuclear IKKα have been described (13–16). We investigated the effects of elevated CO2 on nuclear localization of IKKα in primary human PBMCs exposed to 10% CO2 (Fig. 1E).
activation of the canonical NF-κB signaling pathway in response to activation by LPS. Exposure to 10% atmospheric CO2 inhibited LPS-induced nuclear translocation of the p65 subunit of NF-κB, which was coincident with nuclear translocation of IKKα (Fig. 2A). Furthermore, in wild type MEFs, 10% CO2 suppressed LPS-induced NF-κB activity as determined by a luciferase reporter assay (Fig. 2B).

IKKα is an active component of the IKKα/β/γ signaling complex, but can also exist as a homodimer and can contribute to noncanonical NF-κB signaling (11). We investigated whether the IKKα response to CO2 is dependent on its association with IKKβ. IKKα/β double knockout MEFs (17) were reconstituted with catalytically active IKKα (18). IKKα reconstitution in these cells confers sensitivity of the NF-κB–Luc construct to LPS (Supplemental Fig. 2). In IKKα-reconstituted cells, 10% CO2 significantly attenuated LPS-induced NF-κB activity (Fig. 3A). Furthermore, in IKKα-reconstituted MEFs, there is a robust nuclear localization of IKKα protein in response to CO2 (Fig. 3B). These data demonstrate that in MEFs, the IKKα nuclear localization and inhibition of NF-κB activity is independent of its association with IKKβ.

The signaling consequences of changes in atmospheric CO2 have been best characterized in flies and nematodes. In Drosophila, CO2 is detected by a mechanism involving Gr21a and Gr63 chemosensory receptors that transduce this input into the physiologic response of CO2 avoidance (4). The guanyl cyclase pathway has also been demonstrated to be important in CO2 avoidance, as demonstrated in nematodes that have mutations in Tax-2 and Tax-4 cGMP gated channels (5). In rodents, olfactory sensory neurons detect CO2 via carbonic anhydrase mediated-catalysis of CO2 into bicarbonate, which in turn signals through guanyl cyclase D (6). Interestingly, the role of carbonic anhydrase in acute CO2 sensing is also evident in plants (19, 20). Pseudohypha development in response to CO2 in Candida albicans is absent in mutants lacking adenylyl cyclase, which is thought to act downstream of carbonic anhydrase after detection of CO2-bicarbonate (21). There is evidence from lower species that acute CO2 sensing is mediated through chemosensory neurons, in part via carbonic anhydrase, guanylyl cyclase, and adenylyl cyclase (Fig. 4A).

We investigated whether similar pathways contribute to the IKKα–dependent modulation of the NF-κB pathway by CO2 in mammalian cells. MEFs pretreated with inhibitors of carbonic anhydrase (acetazolamide, 10–500 μM) or guanylyl cyclase (1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1 [ODQ], 100 μM) or an adenylyl cyclase agonist (forskolin, 10 μM) were without significant effect on the CO2-induced nuclear accumulation of IKKα.

**FIGURE 3.** Elevated CO2 attenuates LPS-induced NF-κB activity independent of IKKβ. A, IKKα/β−/− MEFs were reconstituted with catalytically active FLAG-tagged IKKα, transfected with an NF-κB-Luc reporter plasmid and exposed to ambient conditions or 10% CO2 for 24 h with or without LPS treatment (10 μg/ml; 24 h). Reporter activity was analyzed by luminometry (n = 3 ± SEM, ANOVA). B, IKKα/β−/− MEFs were transiently transfected with catalytically active FLAG-tagged IKKα and exposed to ambient conditions or 10% CO2 (4 h). Cytosolic and nuclear extracts were prepared and immunoblotting was performed using the indicated Abs.

**FIGURE 4.** CO2-stimulated nuclear IKKα translocation is not inhibited by acetazolamide, ODQ, and forskolin. A, Schematic summarizing the signaling pathways involved in acute CO2 sensing in lower species (relevant references in italics, inhibitors in red). B, MEFs were pretreated with acetazolamide or ODQ for 1 h prior to exposure to ambient conditions or 10% CO2 (1 h). Acetazolamide, ODQ, and forskolin were added to the media after media change at the time of exposure. Nuclear extracts were prepared and immunoblotting was performed using the indicated Abs.
These data suggest the existence of an intracellular CO₂ sensor pathway linked to IKKα nuclear translocation and suppression of NF-κB, which is independent of pathways that mediate acute CO₂ sensing in lower species.

Changes in CO₂ are inextricably linked to changes in intracellular and extracellular pH. We next investigated whether changes in extracellular pH contribute to CO₂-induced IKKα nuclear localization. Culture media for use at ambient (0.03%) CO₂ were adjusted with concentrated HCl and equilibrated overnight to pH-match it with osmolarity-corrected culture media for use at 10% CO₂. Under these conditions of tightly buffered extracellular pH (Fig. 5A), MEFs were exposed to 0.03 or 10% CO₂ for 4 h ± LPS (10 µg/ml) for the final hour. Despite the buffered pH, IKKα accumulated in the nuclear fraction after exposure to 10% CO₂. Suppression of LPS-induced p65 nuclear accumulation and cytoplasmic IκBα degradation in hypercapnia was also observed (Fig. 5B, 5C). This effect is also evident at more moderate (5%) CO₂ levels (Supplemental Fig. 3). Our findings are consistent with recent studies in Drosophila that provide evidence for hypercapnia-mediated immunosuppression independent of acidosis (9).

Similarly, exposure of MEF to medium designed to have either neutral or acidic pH values—achieved through the addition of sodium bicarbonate and osmolarity balanced using equiosmolar NaCl (Fig. 6A–D)—had no effect on the nuclear localization of IKKα at ambient CO₂ levels, whereas 10% CO₂-induced IKKα nuclear localization was no different between neutral or acidic media (Fig. 6A, 6B). The insensitivity of IKKα to extracellular pH change is further demonstrated over a wider range of pH values (Supplemental Fig. 4). Furthermore, there was no significant difference in intracellular pH detected in MEFs exposed to

**FIGURE 5.** Elevated CO₂ induces IKKα nuclear translocation when extracellular pH is buffered. A, MEFs were exposed to ambient conditions or 10% CO₂ for 4 h ± LPS treatment (10 µg/ml, 1–4 h) with buffered extracellular pH. Media pH was recorded at the start of the experiment and in media taken from the cells at the end (n = 3 ± SEM). Cytosolic (B) and nuclear (C) extracts were prepared and immunoblotting was performed using the indicated Abs.

**FIGURE 6.** Alterations in extracellular or intracellular pH do not promote IKKα nuclear translocation. MEFs were exposed to a range of extracellular media-pH conditions: neutral (N; 0.03% CO₂ [pH 7.54], 10% CO₂ [pH 7.44]) or acidic (A; 0.03% CO₂ [pH 7.05], 10% CO₂ [pH 7.08]) under ambient conditions or at 10% CO₂ for 1 h. Cytosolic (A), nuclear (B), and whole cell (C) extracts were prepared, and immunoblotting was performed using the indicated Abs. D, Intracellular pH measurements using BCECF fluorescence in MEFs exposed to the above conditions (n = 3 ± SEM).
these conditions that can account for the nuclear localization of IKKα at 10% CO₂ (Fig. 6D). Based on these data, we hypothesize that elevated CO₂ affects NF-κB signaling in a manner that is independent of changes in extracellular or intracellular pH.

Finally, to investigate the effects of this response in terms of altered gene expression, we investigated the effect of elevated CO₂ on NF-κB-dependent gene expression. We exposed A549 cells to ambient or 10% CO₂ for 4 h ± the NF-κB ligand lymphopxin-α1/β2 (LT; 100 ng/ml; using buffered media). Cell viability was not affected under these conditions (Supplemental Fig. 7). cDNA generated from these cells was assayed on a PCR array with 84 genes with known associations to the NF-κB signaling pathway. A number of genes differentially regulated at 10% CO₂ were selected for validation. These genes include the chemokine CCL2 (MCP1), the adhesion molecule ICAM1, the proinflammatory cytokine TNF-α, and the anti-inflammatory cytokine IL-10 (Fig. 7A–D). The full array data are included as Supplemental Table I and include evidence for a global effect of CO₂ on the NF-κB transcriptional response. Other genes of interest that were differentially regulated in the array include TLR family members and other inflammatory mediators such as IL-6, which has recently been described to be affected by elevated CO₂ in macrophages (22).

Interestingly, the expression of the genes associated with a proinflammatory response (CCL2, ICAM1, and TNF-α) were blunted at 10% CO₂, whereas expression of IL-10, which is known to have anti-inflammatory properties (23), was enhanced at 10% CO₂ in the presence of LT (Fig. 7A–D). Furthermore, using two different ligands in a different cell line (MEF), we observe that LPS- and TNF-α–induced induction of CCL2 was significantly blunted at 10% CO₂ when compared with ambient CO₂ under pH buffered conditions (Supplementary Fig. 5). These data are strongly supportive of CO₂-mediated transcripational changes that result in an anti-inflammatory, immunosuppressive phenotype.

The contribution of IKKα to the suppression of NF-κB signaling at 10% CO₂ was next investigated using an siRNA-based approach. A549 cells with and without IKKα siRNA treatment were exposed to ambient or 10% CO₂ with and without LT or TNF-α. Hypercapnia clearly affects the cytoplasmic expression of inhibitory protein IκBα in the presence and absence of a ligand in cells treated with nontarget siRNA. Interestingly, the effect of 10% CO₂ on IκBα is still evident in cells treated with IKKα siRNA (Supplemental Fig. 6). Thus, it appears that although the cellular localization of IKKα is profoundly influenced by CO₂, it might not be directly responsible for the suppressive effects on NF-κB signaling observed further downstream. An important caveat, however, is that IKKα has a role as a positive regulator of NF-κB signaling as part of the IKK complex and as part of the noncanonical signaling pathway. Furthermore, the knockdown of IKKα by siRNA, although significant, is not a complete knock-down. This can allow for residual IKKα to translocate to the nucleus at 10% CO₂ and elicit suppressive effects on NF-κB target genes, which have been described previously (13, 15, 16). It appears that there are at least two inputs for CO₂ sensitivity within the NF-κB pathway. The first is the profound rapid, reversible, dose-dependent nuclear localization of IKKα that occurs in response to elevated CO₂ (1–10%). The second is at the level of IκBα at which cytoplasmic expression of this inhibitory protein is maintained or enhanced against a background of elevated CO₂ (5–10%), which likely attenuates downstream NF-κB target gene expression.

**Discussion**

Traditionally, CO₂ has been considered a waste product of respiration, and its biologic activity is poorly understood in terms of gene expression. However, a recent study reported differential gene expression in elevated CO₂ (9). Furthermore, CO₂ has been implicated in development, motility, and lifespan in *Caenorhabditis elegans* (24).

Canonical NF-κB signaling is characterized by activation of the IKKα/β/γ signalsome by upstream adaptor molecules in response to ligand binding. The IKK complex is responsible for the phosphorylation and degradation of the inhibitory protein IκBα, which allows for the nuclear translocation of p65 and p50 NF-κB dimers to regulate gene expression. However, in response to elevated CO₂, the nuclear localization of both IκBα and IKKα is blunted, resulting in an anti-inflammatory, immunosuppressive phenotype.

**FIGURE 7.** Hypercapnia promotes an anti-inflammatory profile of gene expression. A PCR array of genes known to be involved in the NF-κB signaling cascade was performed on A549 cells exposed to ambient or 10% CO₂ ± LT (100 ng/ml) for 4 h. A selection of differentially expressed genes from the array were chosen for validation. CCL2 (A), ICAM1 (B), TNF-α (C), and IL-10 (D) message levels were determined by quantitative real-time PCR and expressed as a percentage of LT-induced gene expression at 0.05% CO₂ (n = 3 ± SEM, one-way ANOVA, Tukey post-test).
to a ligand. IKKα has a nuclear localization sequence in the N-terminal domain (14), and nuclear IKKα may act as a counterbalance to the proinflammatory signaling of IKKβ (25). Furthermore, nuclear IKKα may mediate cytokine-induced histone phosphorylation at specific promoters, thus modifying histone function and inflammatory gene expression (13, 15), (16).

In this study, we provide evidence for rapid, reversible IKKα nuclear localization in a CO2-dependent manner over a range of physiologic CO2 concentrations that is associated with an attenuation of LPS-induced NF-κB signaling and target-gene expression, which is consistent with CO2 affecting IKKα and contributing to the attenuation of inflammation. Whether IKKα is directly modified by CO2 or whether the CO2-sensitivity is conferred by an upstream signaling or adaptor protein has yet to be determined. Furthermore, it appears that the NF-κB pathway may be modified at more than one point by CO2, as evidenced by the preservation of cytoplasmic IkBα in response to ligand stimulation at elevated CO2 (5–10%). This finding is consistent with observations made in hypercapnic acidosis by Takeshita et al. (12), but different from a recent article from Wang et al. using hypercapnia (22). It is clear that there are profound immune and inflammatory signaling consequences for NF-κB target gene expression against a background of elevated CO2 (10%). Our observations are consistent with those made by both Takeshita et al. (12) and Wang et al. (22). In mammalian models, the beneficial immunomodulatory effects of CO2 have been demonstrated in acute respiratory distress syndrome, hypercapnic acidosis, and sepsis (7, 8, 26). In Drosophila, increased susceptibility to bacterial infection at elevated CO2 is independent of pH change (9). Furthermore, increased susceptibility to infection persists in rats with normal renal buffering of CO2 by CO2 or whether the CO2-sensitivity is conferred by an upstream signaling or adaptor protein has yet to be determined.

References


Supplementary Figure 1.

A.  

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>1</th>
<th>2 hrs@ 1% O₂, 5% CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>IKKα</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IKKα</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IKKα</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.  

Minutes of hypoxia 1% O₂ 5% CO₂

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IKKα</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C.  

<table>
<thead>
<tr>
<th></th>
<th>110</th>
<th>39</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂</td>
<td>mmHg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>H</th>
<th>R</th>
<th>N</th>
<th>H</th>
<th>R</th>
<th>N</th>
<th>H</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IKKα</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Start</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21</td>
<td>14</td>
<td>21</td>
<td>21</td>
<td>5</td>
<td>21</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>End</td>
<td>.03</td>
<td>.03</td>
<td>.03</td>
<td>.03</td>
<td>.03</td>
<td>.03</td>
<td>.03</td>
<td>.03</td>
<td>.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21</td>
<td>14</td>
<td>21</td>
<td>21</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>14</td>
<td>21</td>
<td>21</td>
<td>5</td>
<td>21</td>
</tr>
</tbody>
</table>
Figure S1. (A) Multiple cell types (MEF, CCD-19Lu, Hela and HK-2) demonstrate accumulation of IKKα in the nuclear fraction following exposure to 1% O₂ / 5% CO₂ (1-2hrs). (B) MEF were exposed to 1% O₂ / 5% CO₂ for 0-90 minutes and nuclear translocation of IKKα was determined by western blot. (C) MEF were exposed for 1hr to control conditions (C; 21% O₂, 5% CO₂) or 25-110 mmHg O₂ / 5%CO₂) without (H) or with (R) re-equilibration to ambient conditions for 10mins. For A, B and C nuclear extracts were prepared and immunoblotting was carried out using the indicated antibodies. (D) MEF were exposed for 1hr to 1.2 or 10 % CO₂ without or with (R) re-equilibration to ambient conditions for 5mins. Cytosolic and nuclear extracts were prepared and immunoblotting was carried out using the indicated antibodies.
**Supplementary Figure 2.**

*Figure S2.* IKKα−/− MEF were transiently transfected with an NF-kappaB-Luc promoter-reporter vector or control pcDNA plasmid with or without catalytically active Flag-tagged IKKα and treated with or without LPS (10μg/ml; 24hrs).
**Figure S3. NF-κB signalling is attenuated at 5% CO₂**. MEF were exposed ambient or 5% CO₂ for 4hrs +/- LPS (10μg/ml) for the final hour. (A) Cytosolic and (B) nuclear extracts were prepared and immunoblotting was carried out using the indicated antibodies.
Figure S4. Altered extracellular pH does not promote IKKα nuclear translocation. (A) MEF were exposed to a range of extracellular media-pH conditions under ambient conditions or at 10% CO₂ for 1hr (n=3+/− SEM). (B) Cytosolic and (C) nuclear extracts were prepared and immunoblotting was carried out using the indicated antibodies. Results are representative of 3 independent experiments.
Supplementary Figure 5.

Figure S5. Hypercapnia significantly attenuates ligand-induced CCL2 gene expression. MEF were exposed to ambient conditions or 10% CO₂ for 8hrs with or without LPS (A; 10μg/ml, 4hrs) or TNFα (B; 10ng/ml, 8 hrs). mRNA was extracted and qRT-PCR performed using CCL2 specific primers. Results are expressed as % of stimulus-induced CCL2 mRNA. n=3 +/- SEM, 1-way ANOVA, Tukey post-test.
Figure S6. 10% CO₂ maintains cytosolic IκBα in the presence of IKKα siRNA. A549 cells were transfected with 40nM Non-target (NT) or IKKα siRNA for 48 hrs prior to exposure to ambient or 10% CO₂ for 4hrs +/- LT (100ng/ml) or TNFα (10ng/ml) for the final hr of exposure. Cytosolic extracts were prepared and immunoblotted using the indicated antibodies.
Figure S7. Cell viability is not affected by exposure to 10% CO₂. A549 cells were exposed ambient or 10% CO₂ for 4hrs. Cells were trypsinised and incubated with Tryptan blue dye. Tryptan blue negative cells were scored as viable. Experiments were carried out in duplicate for n=3 experiments. Mean +/- SEM.